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APPLICATION

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TITLE: PLANTS CONTAINING A CYTOSOLIC ACETYL COA-CARBOXYLASE NUCLEIC ACID

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Plants Containing a Cytosolic Acetyl CoA-Carboxylase Nucleic Acid

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119(e) of U.S. provisional application serial number 60/198,794, filed April 20, 2000.

TECHNICAL FIELD

This invention relates to oilseed plants, and more particularly to plants containing a nucleic acid encoding a cytosolic acetyl coA-carboxylase (ACCase) enzyme.

BACKGROUND

Acetyl-CoA carboxylase [ACCase; EC 6.4.1.2] catalyzes the first committed step in fatty acid biosynthesis by converting acetyl-CoA to malonyl-CoA. In plants, a multisubunit (MS) form and a multifunctional (MF) form of ACCase have been identified. The MS form is composed of dissociable subunits of different sizes, including a biotin carboxyl carrier protein (BCCP), α- and β-carboxyltransferases (α-CT and β-CT, respectively), and a biotin carboxylase (BC). The MS form is present in plastids of dicotyledenous and of non-*Gramineae* monocotyledenous plants and is primarily involved in the biosynthesis of fatty acids.

The MF form of a plant ACCase is similar to mammalian ACCase (and is sometimes designated "eukaryotic" or "cytosolic" ACCase), in that it is a MF polypeptide with a molecular weight of more than 200 kDa. The MF form of ACCase from plants contains BCCP, BC, α-CT and β-CT functional domains in a single polypeptide. MF ACCase is most likely present in the cytosol of all plant species and in the chloroplasts of *Gramineae* plants. Plant MF ACCase is involved in the biosynthesis of very long chain fatty acids, flavonoids, and in the malonation of amino acids and aminocyclopropane-1-carboxylate (a precursor to ethylene).

Antisense nucleic acids against an MF ACCase have been introduced into *Brassica napus* (White *et al.*, 1998, in *Adv. in Plant Lipid Res.*, pp. 62-66, eds., Sánchez, J., Cerdá-Olmedo, E. & Martinez-Horce, E., Universidad De Sevilla, Spain) and an *Arabidopsis*

genomic DNA encoding an MF ACCase under the control of a napin seed-specific promoter and linked to a small subunit (ss) Rubisco transit peptide was introduced into *B. napus* (Roesler *et al.*, 1997, *Plant Physiol.*, 113:75-81; US Patent No. 5,925,805).

SUMMARY

Plants have been engineered to express a nucleic acid encoding an MF acetyl coA-carboxylase (ACCase), hereinafter referred to as cytosolic ACCase. Oil content was significantly increased in plants containing the cytosolic ACCase coding sequences.

In general, the invention feature plants containing a nucleic acid construct carrying a nucleic acid encoding a cytosolic ACCase operably linked to a promoter and lacking a transit peptide. This plant produces seeds that exhibit a statistically significant increase in oil content as compared to seeds produced by a corresponding plant lacking such a construct.

The invention additionally features plants containing a nucleic acid construct carrying a nucleic acid encoding a cytosolic ACCase lacking introns operably linked to a promoter. This plant produces seeds that exhibit a statistically significant increase in oil content as compared to seeds produced by a corresponding plant lacking such a construct.

The invention also features methods of producing a transgenic plant. This method includes selecting progeny transgenic plants of a plant containing a nucleic acid construct carrying a nucleic acid encoding a cytosolic ACCase operably linked to a promoter. Following at least one generation of selection, one or more of the progeny transgenic plants produce seeds exhibiting a statistically significant increase in oil content as compared to seeds produced by a corresponding plant lacking such a construct.

The invention further features methods of producing a plant by introducing a construct carrying a nucleic acid encoding a cytosolic ACCase operably linked to a promoter into one or more plants. Progeny of these plants, following at least one generation of selection, produce seeds that exhibit a statistically significant increase in oil content when compared to seeds produced by a corresponding plant lacking such a construct.

Yet another feature of the invention are methods of increasing the oil content in seeds by creating a plant containing a nucleic acid construct carrying a gene encoding a cytosolic ACCase operably linked to a promoter; and selecting progeny of the plant that exhibit a

statistically significant increase in oil content in seeds as compared to seeds produced by a corresponding plant lacking such a construct.

Additionally featured in the invention are seeds produced by the above-described plants, and progeny of those plants, wherein the progeny produce seeds that exhibit a statistically significant increase in oil content when compared to seeds produced by the progeny of plants lacking such a construct.

Typically, the increase in oil content is from about 5% to about 25% on a dry weight basis. The above-described selection steps can include selecting progeny that contain the nucleic acid construct. Generally, soybean plants or *Brassica* plants, for example, *Brassica napus*, *B. rapa*, *B. juncea*, *B. carinata*, *B. nigra* and *B. oleracea* are useful in the invention.

Still yet another feature of the invention is a nucleic acid construct carrying a cytosolic ACCase coding sequence operably linked to a promoter but lacking a transit peptide and a nucleic acid construct carrying a cytosolic ACCase coding sequence lacking introns operably linked to a promoter.

A promoter included in a construct of the invention can be a cauliflower mosaic virus (CaMV) 35S promoter. Unless otherwise indicated, the ACCase constructs described herein may or may not include nucleic acid sequences encoding a transit peptide operably linked to the nucleic acid sequences encoding the cytosolic ACCase. An example of a transit peptide is a tobacco small subunit Rubisco transit peptide. In addition, a nucleic acid encoding a cytosolic ACCase can encode a plant cytosolic ACCase, for example, an alfalfa cytosolic ACCase. Further and unless otherwise indicated, a nucleic acid encoding the ACCase can lack introns.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. The materials methods, and examples are illustrative only and not intended to be limiting. Suitable methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the drawings and detailed description, and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 is the nucleotide and amino acid sequence (SEQ ID NO:3 and 4 respectively) of the tobacco small subunit (ss) Rubisco transit peptide and 5' portion of the mature ss Rubisco protein (underlined).

Figure 2 is a representative +6ACCase construct (SEQ ID NO:5). The nucleotide sequence encoding a transit peptide and the 5' portion of a small subunit (ss) Rubisco gene from tobacco is shown operably linked to an alfalfa cytosolic ACCase coding sequence. A consensus sequence for initiation of translation is italicized and includes the 3' end of a 35S cauliflower mosaic virus (CaMV) promoter and the 5' sequence encoding the tobacco ssRubisco transit peptide. The ACCase sequence corresponds to a portion of the coding sequence and 3' untranslated sequences (See Genbank Accession No. L25042); for the entire ACCase coding sequence). Arrows indicate the methionine-initiated (M) start codon of the ssRubisco transit peptide, the beginning of the portion of the ssRubisco mature protein included in the construct, the beginning and end of the ACCase coding sequence as published in GenBank, and the end of the ACCase 3' untranslated sequences. The *Bam*HI and *Kpn*I restriction sites were used to clone the +6ACCase construct into the ptet vector.

Figure 3 is a representative –7ACCase construct (SEQ ID NO:6). The italicized consensus sequence for the initiation of translation includes the 3' end of a 35S cauliflower mosaic virus (CaMV) promoter and the 5' portion of an alfalfa cytosolic acetyl coA-carboxylase (ACCase) coding sequence (Shorrosh et al., 1994). The ACCase sequences are as described in the legend to Figure 2. Arrows indicate the methionine-initiated (M) start codon, the end of the ACCase coding as published in GenBank and the end of the ACCase 3' untranslated sequences. The *Bam*HI and *Kpn*I restriction sites were used to clone the -7ACCase construct into the ptet vector.

Figure 4 is the nucleotide and amino acid sequence (SEQ ID NO:7 and 8, respectively) of an alfalfa cytosolic acetyl coA-carboxylase (ACCase) (GenBank Accession No. L25042 plus additional 3' untranslated sequences).

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

All percent oil content and percent protein content are reported based upon dry weight. As used herein, "oil content" or "percent oil content" refers to the oil content in a particular tissue. "Oils" are typically triacylglycerols. Oil content can be measured in by NMR (using American Oil Chemists' Society (AOCS) Method AM 2-93 and AOCS Recommended Practice AK 4-95) or by NIR (using AOCS Method AK 3-94 and AOCS Procedure AM 1-92).

As used herein, "protein content" or "percent protein content" refers to the protein content in a particular tissue. The protein content in seeds typically includes storage proteins, as well as other peptide/polypeptide components. Protein content can be determined by NIR (using AOCS Method BA 4e-93).

As used herein, "high oleic acid" refers to an oleic acid ($C_{18:1}$) content in seeds greater than 70% based on total fatty acid composition after hydrolysis. A typical high oleic *Brassica* line exhibits an oleic acid content of at least 70%; for example, an oleic acid content of about 80%, or about 90% based on total fatty acid composition after hydrolysis. Oleic acid is typically measured by gas chromatography (GC) using AOCS Method Ce 1e-91.

As used herein, "high erucic acid" refers to an erucic acid ($C_{22:1}$) content greater than 45% based on total fatty acid composition after hydrolysis. A typical high erucic acid *Brassica* line would exhibit an erucic acid content of at least 45%; for example, an erucic acid content of 50%, 55% or even greater based on total fatty acid composition after hydrolysis. Erucic acid is typically measured by GC using AOCS Method Ce 1e-91.

As used herein, "FDA saturated fatty acid content" is the total of myristate ($C_{14:0}$), palmitate ($C_{16:0}$), stearate ($C_{18:0}$), arachidate ($C_{20:0}$), behenate ($C_{22:0}$) and lignocerate ($C_{24:0}$). Fatty acid profiles reported herein were obtained by GC (using AOCS Method Ce 1e-91).

As used herein, a "variety" is a group of plants that display little or no genetic variation between individuals for at least one trait. Varieties may be created by, *e.g.*, several

generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques.

As used herein, a "line" refers to a plant and its progeny produced from a single transformation.

Nucleic acid constructs

A nucleic acid construct useful in the invention comprises a multi-functional cytosolic acetyl coA-carboxylase (ACCase) coding sequence operably linked to a promoter. Suitable cytosolic ACCases include plant and animal cytosolic ACCases from organisms such as *Arabidopsis thaliana* (*e.g.*, GenBank Accession No. L27074), *Brassica napus* (*e.g.*, GenBank Accession No. X77576), *Zea mays* (*e.g.*, GenBank Accession No. A25273) and *Homo sapiens* (*e.g.*, GenBank Accession No. U19822). For example, a construct can contain a 35S cauliflower mosaic virus (CaMV) promoter and an alfalfa (*i.e.*, *Medicago sativa*) cytosolic ACCase cDNA (*e.g.*, GenBank Accession No. L25042).

Alternatively, a construct of the invention can contain ACCase nucleic acid sequences from *Saccharomyces cerivisiae* (*e.g.*, GenBank Accession No. M92156), *Schizosaccharomyces pombe* (*e.g.*, GenBank Accession No. D78169), *Ustilago maydis* (*e.g.*, GenBank Accession No. Z46886), *Bos taurus* (bovine) (*e.g.*, GenBank Accession No. AJ132890), *Rattus norvegicus* (rat) (*e.g.*, GenBank Accession No. AB004329), *Ovis aries* (sheep) (*e.g.*, GenBank Accession No. X80045), *Gallus gallus* (chicken) (*e.g.*, GenBank Accession No. J03541), *Glycine max* (soybean) (*e.g.*, GenBank Accession No. L42814), *Avena sativa* (oat) (*e.g.*, GenBank Accession No. AF072737), *Triticum aestivum* (wheat) (*e.g.*, GenBank Accession No. U39321) or *Phaseolus vulgaris* (bean) (*e.g.*, GenBank Accession No. AF007803). A representative cloning strategy for producing a construct of the present invention is described herein. Other suitable methods for engineering constructs are described elsewhere, *e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press (1989).

As used herein, "promoter" refers to nucleic acid sequences that, when operably linked to an ACCase coding sequence, direct transcription of the coding sequence such that it's gene product can be produced. Promoters can be described based on their activity (*e.g.*, constitutive, inducible, tissue-specific or temporal-specific). Constitutive promoters are

generally nucleic acid sequences that direct a relatively high level of transcription, and typically without great tissue- or temporal-specificity. Inducible promoters are typically nucleic acid sequences that regulate transcription in response to a stimulus (*e.g.*, a physical or chemical stimulus). Tissue- or temporal-specific promoters are generally nucleic acid sequences that direct transcription that is biased toward a particular tissue or time (*e.g.*, a particular developmental stage), respectively. Oftentimes, however, a promoter's activity does not fall under a single description.

Suitable promoters are known (*e.g.*, Weising *et al.*, *Ann. Rev. Genetics* 22:421-478 (1988)). The following are representative promoters suitable for use in the invention described herein: regulatory sequences from fatty acid desaturase genes (*e.g.*, *Brassica fad2D* or *fad2F*, see WO 00/07430); alcohol dehydrogenase promoter from corn; light inducible promoters such as the ribulose bisphosphate carboxylase (Rubisco) small subunit gene promoters from a variety of species; major chlorophyll a/b binding protein gene promoters; the 19S promoter of cauliflower mosaic virus (CaMV); a seed-specific promoter such as a napin or cruciferin seed-specific promoter; as well as synthetic or other natural promoters that are, for example, inducible, constitutive, tissue-specific or temporal-specific.

A nucleic acid construct optionally may contain a nucleic acid sequence encoding a transit peptide operably linked to an ACCase coding sequence. A transit peptide facilitates transport to plastids of the ACCase polypeptide to which the transit peptide is fused. Suitable transit peptides include any transit peptide encoded by a nuclear gene that directs transport of the encoded protein into the chloroplast.

A nucleic acid encoding a cytosolic ACCase may or may not contain introns within the coding sequence. Introns are nucleic acid sequences that are initially transcribed into RNA and subsequently removed. The number of introns in a transcript can vary, as can the size of each intron. Introns themselves possess very little conservation, but the splice site sequences (*i.e.*, the sequence at the exon-intron and intron-exon junctions) typically are highly conserved among eukaryotes. In addition, introns typically possess an internal conserved sequence corresponding to an branch site involved in intron removal. Nucleic acid sequences containing an ACCase open reading frame can be examined for introns using, for example, software such as the Sequence Analysis Software Package of the Genetics Computer Group (GCG) (University of Wisconsin Biotechnology Center, 1710 University

Avenue, Madison, WI 53705). An ACCase nucleic acid having introns can be, for example, a genomic ACCase nucleic acid (*e.g.*, GenBank Accession No. L27074). An ACCase nucleic acid lacking introns can be, for example, a complementary DNA (cDNA) of an ACCase mRNA nucleic acid (*e.g.*, SEQ ID NO:7), or can be assembled (*e.g.*, recombinantly) from individual exonic sequences.

It should be appreciated that many different nucleic acids will encode a polypeptide having a particular cytosolic ACCase amino acid sequence. The degeneracy of the genetic code is well known in the art, *i.e.*, many amino acids are coded for by more than one nucleotide codon. It should also be appreciated that certain amino acid substitutions can be made within polypeptide sequences without affecting the function of the polypeptide. Conservative amino acid substitutions or substitutions of similar amino acids often are tolerated without affecting polypeptide function. Similar amino acids can be those that are similar in size and/or charge properties. Similarity between amino acids has been assessed in the art. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure,* Vol. 5, Suppl. 3, pp. 345-352, incorporated herein by reference, provides frequency tables for amino acid substitutions that can be employed as a measure of amino acid similarity.

Additional regulatory sequences may be useful in the nucleic acid constructs of the present invention, including, but not limited to, polyadenylation sequences, enhancers, introns, and the like. Such elements may not be necessary for expression of the ACCase coding sequence, although they may increase expression by affecting transcription, stability of the mRNA, translational efficiency, or the like. Such elements can be included in a nucleic acid construct as desired to obtain optimal expression of the ACCase nucleic acid in the host cell(s). Sufficient expression, however, may sometimes be obtained without such additional elements. A representative reference describing certain regulatory elements is Weising *et al.*, *Ann. Rev. Genetics* 22:421-478 (1988).

Transgenic plants

In one aspect of the invention, transgenic plants are created by introducing an ACCase nucleic acid construct into a plant cell and growing the plant cell into a plant. Such plants contain and express the ACCase nucleic acid construct. Suitable techniques for introducing nucleic acids into plant cells to create such plants include, without limitation,

Agrobacterium-mediated transformation, viral vector-mediated transformation, electroporation and particle gun transformation. Illustrative examples of transformation techniques are disclosed in U.S. Patent 5,204,253, (describing biolistic transformations), U.S. Patent 6,051,756 (describing biolistic transformation of *Brassica*) and U.S. Patent 5,188,958 (describing *Agrobacterium* transformation). Transformation methods utilizing the Ti and Ri plasmids of *Agrobacterium spp.* typically use binary-type vectors (*e.g.*, ptet1, pBin19) (Walkerpeach et al., in *Plant Molecular Biology Manual*, Gelvin & Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994)).

Techniques are known for the introduction of DNA into dicots as well as monocots, as are the techniques for culturing such tissues and regenerating plants. If cell or tissue cultures are used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art. Suitable dicots include plants such as alfalfa, soybean, rapeseed (high erucic and canola), and sunflower. Monocots that have been successfully transformed and regenerated in the art include wheat, corn, rye, rice, sorghum and asparagus (see, *e.g.*, U.S. Patent Nos. 5,484,956 and 5,550,318).

Preferred species for generating transgenic plants of the present invention include, without limitation, oil-producing species, such as soybean (*Glycine max*), rapeseed (*e.g.*, *Brassica napus*, *B. rapa* and *B. juncea*) (both Spring and Winter maturing types within each species), sunflower (*Helianthus annus*), castor bean (*Ricinus communis*), safflower (*Carthamus tinctorius*), palm (*e.g., Elaeis guineensis*), coconut (*e.g., Cocos nucifera*), meadowfoam (*e.g., Limnanthes alba alba* and *L. douglasii*), cottonseed (*e.g., Gossypium hirsutum*), olive (*e.g., Olea europaea*), peanut (*e.g., Arachis hypogaea*), flax (*e.g., Linum usitatissimum*), sesame (*e.g., Sesamum indicum*) and crambe (*e.g., Crambe abyssinica* or *C. hispanica*). Accordingly, suitable families include, but are not limited to, *Solanaceae*, *Leguminaceae*, *Brassicaceae* and *Asteraceae*. A transgenic plant of the invention typically is a member of a plant variety within the families or species mentioned above.

As used herein, a transgenic plant also refers to progeny of an initial transgenic plant. Progeny includes descendants of a particular plant or plant variety, *e.g.*, seeds developed on a particular plant. Progeny of a plant also includes seeds formed on F_1, F_2, F_3, and subsequent generation plants, or seeds formed on BC_1, BC_2, BC_3, and subsequent generation plants. Seeds produced by a transgenic plant can be grown and then selfed (or out-crossed and

selfed) to obtain plants homozygous for the construct. Seeds can be analyzed to identify those homozygotes having the desired level of expression of a construct. Alternatively, transgenic plants and progeny thereof may be obtained by vegetative propagation of a transformed plant cell (for those species amenable to such techniques).

Transgenic plants can be used in commercial breeding programs for the species of interest or can be crossed or bred to plants of related crop species. Phenotypes conferred by expression of an ACCase nucleic acid construct can be transferred from one species to another species by, for example, protoplast fusion. Such breeding programs are useful to incorporate other agronomic or specialty traits of interest, *e.g.*, herbicide tolerance or a high oleic acid content in seeds.

Methods

In one aspect of the invention, there are provided methods of generating a plant that produces seeds exhibiting a statistically significant increase in oil content. The method includes introducing a nucleic acid construct containing a promoter and an ACCase coding sequence into a plant and selecting progeny that produce seeds with increased oil content as compared to seeds from a corresponding plant lacking the ACCase nucleic acid construct, *e.g.*, seeds from a plant having the same or similar genetic background as the transgenic plant but which does not have the cytosolic ACCase construct. Such progeny are identified after one or more generations of selection, *e.g.*, one generation, three or more generations, or six or more generations. By way of example, selection may be carried out initially, *e.g.*, the first and second generations, by selecting those progeny possessing the ACCase construct, and selection in subsequent generations may be carried out by identifying those progeny that possess the ACCase construct as well as elevated seed oil content.

Also provided by the invention are methods of producing seeds with a statistically significant increase in oil content. The methods include introducing a nucleic acid construct containing a promoter and an ACCase coding sequence into one or more plant cells and regenerating such plant cells into one or more plants. Seeds exhibiting statistically significantly increased oil content can then be harvested from selected progeny of the plant.

Further provided by the invention are methods of increasing the oil content in seeds. The methods include introducing a nucleic acid construct containing a promoter and a

cytosolic ACCase coding sequence into a plant and selecting progeny after at least one generation of selection that produce seed with increased oil content as compared to corresponding seeds produced from plants lacking the recombinant ACCase nucleic acid.

The following Table provides relative percent oil and protein content on a dry weight basis (unless indicated otherwise) in several plants, particularly oilseed plants, that can be used in the present invention.

Plant	% Oil	% Protein	Key
Soybean (*Glycine max*)	~20	~40	c
Rapeseed (*Brassica napus*)	40-44	38-41 (oil free meal)	c; d
Sunflower (*Helianthus annus*)	40		d
Castor bean (*Ricinus communis*)	50		a
Safflower (*Carthamus tinctorius*)	36.8-47.7	15.4-22.5	d
Crambe (*Crambe abyssinica*)	30-35	~28	b
Palm (*Elaeis guineensis*)	20 >50		c; per fresh fruit bunch (~20% moisture); dried kernels
Coconut (*Cocos nucifera*)	34 69	3.5	d; coconut flesh (50% moisture); dried kernels
Maize (*Zea mays*)	3.1-5.7	6-12	c; d
Cottonseed (*Gossypium hirsutum*)	25-30	25-30	d; kernel
Olive (*Olea europaea*)	19.6	1.6	fruit (52.4% moisture)
Peanut (*Arachis hypogaea*)	36-56	25-30	c; (unknown moisture)
Flax (*Linum usitatissimum*)	35-45		d; per fruit capsule (~10 seeds/fruit)
Sesame (*Sesamum indicum*)	53.3-57.5	25-30	d; (5-7% moisture)

(a) Brigham RD, 1993, Castor: Return of an old crop, p 380-3. In *New Crops*, Janick, J & Simon, JE, eds. Wiley, NY.

(b) Grombacher et al., Cooperative Extension, Institute of Agriculture and Natural Resources, University of Nebraska-Lincoln, *Crambe production*, Publication G93-1126A, G1126 (Field Crops), F-17 (Misc. Crops); see also pubs@unlvm.unl.edu

(c) In *Principles of Cultivar Development*, 1987, Fehr, WR, ed., Macmillan Publishing Co., NY.

(d) In *5th Edition Bailey's Industrial Oil & Fat Products*, Vol. 2, Edible Oil & Fat Products: Oils and Oil Seeds, 1996, Hui, YH, ed., Wiley, NY.

The present invention describes a novel method of making plants that produce seeds with a statistically significant increase in oil content. As used herein, "statistically significant" refers to a *p*-value of less than 0.05, *e.g.*, a *p*-value of less than 0.025 or a *p*-value of less than 0.01, using an appropriate measure of statistical significance, *e.g.*, a one-tailed two sample t-test.

Plants of the invention produce seeds that exhibit an increase in oil content that is statistically significant relative to seeds produced by plants that lack a cytosolic ACCase construct. Plants produced by the method of the present invention produce seeds having an increase in oil of from about 5% to about 25% over the oil content in seeds produced by untransformed control plants. For example, the increase in oil content for plants described herein is from about 5% to about 20%, or from about 5% to about 15%, or from about 10% to about 20%, relative to plants that lack a cytosolic ACCase construct.

The seeds of several different Brassica napus lines from a –7ACCase/Westar transformation have been deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA, 20110-2209, and have the following accession numbers.

Line	Accession No.	Deposit Date
Tao-001-30-02		
Tao-001-31-02		
Tao-001-56-01		
Tao-001-56-06		
Tao-001-65-08		

Seeds and plants of plant varieties made from the transgenic plants described herein are included within the scope of the invention, as well as progeny of these varieties that possess the novel characteristics recited herein. Oil extracted from such varieties or from similar varieties is also within the scope of the invention.

Nucleic acid constructs, plants and methods described herein provide for more efficient production of oil for food and industrial applications (*e.g.*, engine lubricants, hydraulic fluids, etc.). For example, plants described herein produce a greater yield of oil per acre planted compared to plants lacking a cytosolic ACCase construct. In addition, there is increased oil yield during the processing of such seeds.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1—Constructs

The pSP72 vector (Promega) was digested with *Xho*I and *Sal*I and subsequently religated to remove the *Pvu*II site. This modified vector was designated ModpSP72. The tobacco small subunit Rubisco (ssRubisco, also known as ribulose 1,5-bisphosphate carboxylase) transit peptide was amplified by PCR from a tobacco ssRubisco gene/pet11d template using a 5' primer (5'-CAUCAUCAUCAUATCGATAGGTACCAAAAAAAA CAACCATGGCTTCCTCAGTTCTT) (SEQ ID NO:1) and a 3' primer (5'-CUACUAC UACUAGCTAGCCATGGACT TCTTGTTAATTGGTGGCCA) (SEQ ID NO:2). The 5' primer was designed to contain *Cla*I, *Kpn*I, and *Nco*I sites, and the 3' primer was engineered to contain *Nco*I and *Nhe*I sites. The amplified transit peptide DNA was annealed into the pAMP1 vector (Gibco BRL) and both strands were sequenced to confirm fidelity. This construct was designated +Transit/pAMP1. To generate a construct lacking the transit peptide (–Transit/pAMP), +Transit/pAMP1 was digested with *Nco*I and religated.

The cloning of a fragment designated 209/180 from an alfalfa cytosolic acetyl coA-carboxylase (ACCase) into the pAMP1 vector to produce 209/180/pAMP1 is described in Shorrosh et al. (1994, *Proc. Natl. Acad. Sci. USA*, 91:4323-27). A fragment designated 147/136 was PCR amplified using primers 147 and 136 (Shorrosh et al., 1994), which was subsequently subcloned into the pAMP1 vector to generate a 147/136/pAMP1 construct. The 209/180 fragment was removed from the pAMP1 vector by digesting with *Kpn*I and *Bam*HI and subcloned into the *Kpn*I/*Bam*HI sites of ModpSP72 to generate a 209/180/pSP72 construct. The 147/136/pAMP1 construct was digested with *SnaB*I and *Bam*HI and the insert

containing the 147/136 fragment was subcloned into the *SnaBI*/*Bam*HI sites of the 209/180/pSP72 construct to generate a 209/136/pSP72 construct.

Clone "T1", corresponding to a partial alfalfa ACCase cDNA and described in Shorrosh et al., 1994, was digested with *Pvu*II and *Bam*HI and subcloned into the 209/136/pSP72 construct at the *Pvu*II/*Bam*HI sites to generate 209-T/pSP72. Additionally, a clone designated 3X, corresponding to a partial alfalfa ACCase cDNA (essentially the M2 fragment as described in Shorrosh et al., 1994, with additional 5' and 3' flanking sequences to facilitate cloning), was digested with *Eco*R47III and *Bam*HI and subcloned into the 209-T/pSP72 construct at the *Eco*R47III/*Bam*HI sites to generate 209-3X/pSP72. This construct contains a full-length alfalfa cytosolic ACCase cDNA coding sequence in the pSP72 vector.

The 209-3X/pSP72 construct was digested with *Kpn*I and *Bam*HI and subcloned into the +Transit/pAMPI construct at the *Kpn*I/*Bam*HI sites to generate a construct designated +6ACCase/pAMP1. +6ACCase/pAMP1 contains a full-length alfalfa ACCase cDNA with a transit peptide at the 5' end in the same reading frame as the ACCase coding sequence. The +6ACCase/pAMP1 construct was then digested with *Nhe*I and *Bam*HI and the full-length alfalfa ACCase cDNA, including the transit peptide, was subcloned into the *Agrobacterium* binary vector, ptet1 (provided by Dr. C. Gatz, Institute fur Genbiologische, Berlin), at the *Nhe*I/*Bam*HI sites adjacent to the cauliflower mosaic virus (CaMV) 35S promoter. This manipulation generated +6ACCase/ptet1. Similarly, the 209-3X/pSP72 construct was digested with *Nhe*/*Bam*HI and the full-length alfalfa ACCase cDNA was subcloned into the -Transit/pAMP1 construct at the *Nhe*I/*Bam*HI sites to generate -7ACCase/pAMP1. The -7ACCase/pAMP1 construct was then digested with *Kpn*I and *Bam*HI and the full-length alfalfa ACCase cDNA was subcloned into the ptet1 binary vector at the *Kpn*I/*Bam*HI sites adjacent to the CaMV 35S promoter to produce -7ACCase/ptet1. The -7ACCase/ptet1 construct contains a full-length alfalfa ACCase cDNA but lacks a transit peptide.

Example 2—Transgenic plants

The +6ACCase/ptet1 and -7ACCase/ptet1 constructs of Example 1 were used to transform *Agrobacterium* LBA4404. The resulting *Agrobacterium* transformants were each co-cultivated separately with *B. napus* hypocotyls and cultured consecutively on incubation,

selection (containing kanamycin) and regeneration media until green shoots were produced. Regenerated plantlets were transferred to the greenhouse and grown to maturity. Each T1 plant (N=240) was selfed and the resulting T2 seeds were harvested from each individual T1 plant.

The ACCase constructs were introduced into *B. napus* hypocotyls of three different canola varieties as follows. A construct designated -7ACCase was introduced into Westar, a canola variety registered in Canada; and a construct designated +6ACCase was introduced into Oscar, a canola variety registered in Australia (App. No. 1992/009, 19 June, 1996) or IMC 03, a Cargill proprietary low linolenic acid canola variety. Table 1 shows a typical fatty acid profile for each of the *Brassica* varieties used in the transformations.

TABLE 1

Typical fatty acid profile of Westar, Oscar and IMC 03 seeds

Fatty acid	Westar	Oscar	IMC 03
$C_{16:0}$	3.7[1]	3.7	3.9
$C_{16:1}$	0.1	0.1	0.1
$C_{18:0}$	2.5	2.5	1.95
$C_{18:1}$	65.0	60	65.6
$C_{18:2}$	17.6	22.0	18.0
$C_{18:3}$	8.0	10.0	3.00
$C_{20:0}$	0.5	0.5	0.5
$C_{20:1}$	1.3	1.3	1.5
$C_{20:2}$	0.1	0.1	0.1
$C_{22:0}$	0.1	0.1	0.1
$C_{22:1}$	0.1	0.1	0.05
$C_{24:0}$	0.1	0.1	0.1
$C_{24:1}$	0.1	0.1	0.1
FDA	6.9	6.9	6.55
% Oil	45.0	41.0	46.0
% Protein	26.97[2,3]	--	22.46[3]

[1]percent; [2]from *Principles in Cultivar Development*, Vol. 2, Crop Species, W.R. Fehr, ed., pp. 443; [3]percent protein content was estimated based upon the percent protein content reported for the air-dried, oil-free seed meal using the oil content reported and assuming a 5% moisture content; --, Unknown.

Example 3—Preparation of fatty acid methyl esters and fatty acid analysis of seed by capillary gas liquid chromatography (GLC)

The following describes a means for quantifying fatty acid composition in canola seed. To prepare samples, approximately 150 mg of seed is placed into a 15 ml polypropylene centrifuge tube. The seed is broken apart and 0.6 ml of methanolic KOH solution is added to the tube. After mixing on a vortex mixer for approximately 30 sec, the tube is placed in a water bath at 60°C for 60 sec. About 4.0 ml of saturated NaCl solution is added to the tube followed by 1.0 ml of iso-octane and the sample mixed on a vortex mixer for an additional 30 sec. The sample is centrifuged for 5 min to separate and purify the organic layer. Approximately 700 µl of the organic layer, which contains the fatty acid methyl esters, is removed from the tube and placed into a GC autosampler vial. The vial is purged with nitrogen gas to remove the oxygen and preserve the sample.

Samples are analyzed, based on AOCS Method Ce 1e-91, by injecting 1.0 µl into a Hewlett Packard 6890 gas chromatograph by means of an autosampler. A normalized percentage is calculated and reported for each fatty acid in the sample.

The GC conditions are as follows:

Column: 5 m x 0.32 mm DB-Wax (0.5 µm film thickness);

Detector: FID;

Inlet temp.: 250°C;

Detector temp.: 250°C;

Split ratio: 100:1;

Carrier gas: helium at 30.0 ml/min; and

Oven program: 1.0 min at 220°C; 10°C/min up to 245°C; and 3.0 min at 245°C.

Example 4—Determination of oil and moisture content in canola seed by NMR spectroscopy

The following is a non-destructive method for determining oil and moisture content in samples of canola seed by means of nuclear magnetic resonance spectroscopy. An Oxford MQA6005 NMR Analyzer (Oxford Analytical Instruments Limited, Concord, MA) is

calibrated according to manufacturer specifications. Six samples of canola seed (~22 g/sample) are used for calibration. Samples are selected to represent the oil and moisture ranges over which most unknown samples are expected to fall. The oil content of each sample is determined by Soxhlet extraction (based on AOCS Method Am 2-93). Moisture content is determined by gravimetric means (based on AOCS Method Ai 2-75). The response of each sample is then measured on the NMR instrument. Two calibration curves (one for oil and one for moisture) are prepared using the data collected.

Samples containing unknown amounts of oil and moisture are analyzed according to the instrument manufacturer instructions (based on AOCS Recommended Practice Ak 4-95). The response of each sample is collected and stored by a computer. The results are calculated and expressed as "Oil %", "Moisture %", and "Oil % Normalized to Dry Mass" (conversion from Oil % (as is) to Oil % on a dry basis is calculated using the following formula: Oil % (dry) = Oil % (as is)/[1-(Moisture %/100)]).

Example 5—Determination of percent oil, moisture, protein, chlorophyll, and fatty acids by NIR spectroscopy

The following method provides a means of predicting the levels of oil, moisture, protein, chlorophyll, oleic acid ($C_{18:0}$), linoleic acid ($C_{18:1}$), and linolenic acid ($C_{18:2}$) in canola seed samples by means of near infra-red reflectance spectroscopy.

A Foss NIR Systems model 6500 Feed and Forage Analyzer (Foss North America, Eden Prairie, MN) is calibrated according to the manufacturer's recommendations. Canola seed samples, which represented wide ranges of the sample constituents listed above, are collected for calibration. Lab analysis results are determined using accepted methodology (*i.e.*, oil, AOCS Method Ak 3-94; moisture, AOCS Method Ai 2-75; fatty acid, AOCS Method CE 1e-91 and AOCS Method CE 2-66; chlorophyll, AOCS Method CC 13D-55; protein, AOCS Method BA 4e-93; and glucosinolates, AOCS Method Ak 1-92). Instrument response is also measured for each sample. A calibration equation is calculated for each constituent by means of chemometrics. These equations are combined into one computer file and are used for prediction of the constituents contained in unknown canola samples.

Seed samples containing unknown levels of the above constituents are prepared by removing foreign material from the sample. Cleaned whole seed is placed into the

instrument sample cell and the cell is placed into the instrument sample assembly. Analysis is carried out according to instrument manufacturer instructions (based on AOCS Procedure Am 1-92). The results are predicted and reported as % constituent (% oil and protein are reported based on dry weight). Conversion from 'dry weight' basis to 'as is' basis for oil and protein is calculated using the following formula:

constituent (as is) = constituent (dry wt.) x [1-(% moisture/100)].

Example 6—T1 plants and T2 seeds

A total of 126 -7ACCase/Westar plants were regenerated in a greenhouse from the plantlets described in Example 2. Each T1 plant was selfed and a sample of T2 seeds from each plant was analyzed for fatty acid composition by gas chromatography as described in Example 3. T2 seeds had fatty acid compositions that were not significantly different from the fatty acid profile of the Westar background variety.

Table 2 shows the mean fatty acid profile (± standard deviation) for the -7ACCase/ Westar transformation. T2 seeds from each T1 plant were advanced (*i.e.*, no selection was performed on T2 seeds) such that 5-10 seeds from each T1 plant were grown individually in a single row in the greenhouse.

TABLE 2

Mean fatty acid profile of T2 seeds

Fatty acid	-7ACCase/Westar
$C_{14:0}$	0.07 (0.04)[1]
$C_{16:0}$	4.59 (0.69)
$C_{16:1}$	0.31 (0.13)
$C_{18:0}$	2.28 (0.45)
$C_{18:1}$	61.61 (3.53)
$C_{18:2}$	20.18 (2.27)
$C_{18:3}$	7.95 (1.04)
$C_{20:0}$	0.77 (0.12)
$C_{20:1}$	1.23 (0.10)
$C_{20:2}$	0.08 (0.02)
$C_{22:0}$	0.45 (0.09)
$C_{22:1}$	0.03 (0.03)
$C_{24:0}$	0.24 (0.10)
$C_{24:1}$	0.24 (0.17)
FDA	8.39 (1.22)

[1]mean in percent (± standard deviation)

Table 3 shows fatty acid profiles of T2 seeds from representative individual lines from the -7ACCase/Westar transformation.

TABLE 3

Fatty acid profile of T2 seeds from representative individual -7ACCase/Westar *Brassica* lines

	001-01[1]	001-120	001-121	001-31
$C_{14:0}$	0.103[2]	0.448	0.154	0.056
$C_{16:0}$	7.720	7.053	6.339	4.222
$C_{16:1}$	0.776	0.821	0.820	0.249
$C_{18:0}$	3.181	3.107	4.213	1.988
$C_{18:1}$	55.584	51.827	50.624	64.292
$C_{18:2}$	22.659	24.794	25.949	18.319
$C_{18:3}$	6.806	8.343	7.041	8.106
$C_{20:0}$	1.170	0.969	1.235	0.688
$C_{20:1}$	0.950	0.815	0.902	1.337
$C_{20:2}$	0.000	0.065	0.082	0.065
$C_{22:0}$	1.170	0.578	0.912	0.381
$C_{22:1}$	0.000	0.024	0.188	0.000
$C_{24:0}$	0.379	0.421	0.574	0.168
$C_{24:1}$	0.000	0.735	0.966	0.129
FDA	13.225	12.576	13.427	7.504

[1]line designation; [2]%

Example 7—T2 plants and T3 seeds

A total of 834 T2 plants from 126 lines were selfed and the resulting T3 seed analyzed for fatty acid composition by gas chromatography as described in Example 3.

Table 4 shows summary statistics (mean ± standard deviation) of fatty acid profiles of seeds from the total population of T3 plants produced in the -7ACCase/Westar transformation, from those T3 plants selected for advancement and from plants corresponding to the non-transgenic Westar variety. Data for the non-transgenic control plants was obtained from 19 Westar plants grown under similar conditions.

TABLE 4

Mean fatty acid profile of T3 seeds

Fatty acid	-7ACCase/Westar		
	Total[1]	Sel[1]	Control[1]
$C_{14:0}$	0.06[2] (0.02)	0.06 (0.03)	0.06 (0.02)
$C_{16:0}$	4.71 (0.79)	4.90 (1.03)	4.45 (0.51)
$C_{16:1}$	0.27 (0.10)	0.28 (0.13)	0.23 (0.10)
$C_{18:0}$	2.84 (0.68)	3.25 (0.68)	2.68 (0.52)
$C_{18:1}$	66.62 (5.34)	67.20 (6.69)	69.76 (3.41)
$C_{18:2}$	15.67 (3.50)	14.46 (3.88)	13.78 (2.46)
$C_{18:3}$	5.68 (1.43)	5.20 (1.26)	4.90 (1.05)
$C_{20:0}$	1.14 (0.26)	1.32 (0.21)	1.08 (0.29)
$C_{20:1}$	1.38 (0.21)	1.43 (0.24)	1.41 (0.14)
$C_{20:2}$	0.07 (0.02)	0.06 (0.02)	0.05 (0.02)
$C_{22:0}$	0.74 (0.22)	0.87 (0.21)	0.75 (0.16)
$C_{22:1}$	0.03 (0.04)	0.03 (0.04)	0.02 (0.02)
$C_{24:0}$	0.50 (0.16)	0.60 (0.13)	0.55 (0.15)
$C_{24:1}$	0.31 (0.13)	0.34 (0.13)	0.28 (0.06)
FDA	9.99 (1.72)	11.00 (1.84)	9.56 (1.09)

[1]Total, mean composition of all T2 plants; Sel, mean composition of T2 plants selected for advancement; Control, mean composition of non-transformed Westar plants; [2]mean in percent (± standard deviation).

Three hundred ninety-five plots of T2 plants (representing 104 lines) from the -7ACCase/Westar transformation were selected for advancement based on T3 seeds exhibiting one or more of the following properties in fatty acid composition: $C_{18:0}$ >3.45%,

$C_{18:2}$ <13.1%, $C_{18:3}$ <4.51%, $C_{20:0}$ > 1.55%, or FDA saturates (defined as the sum of $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$) >10.5%.

Table 5 shows the fatty acid profile of T3 seed from representative individual lines from the -7ACCase/Westar transformation that were selected for advancement. Bolded numbers indicate criteria used to select and advance the plants.

TABLE 5

Fatty acid profile of T3 seeds from representative -7ACCase/Westar lines selected for advancement

Fatty acid	001-26-01[1]	001-27-12	001-30-02	001-31-05	001-31-07	001-78-04
$C_{14:0}$	0.0755[2]	0.1100	0.0371	0.0393	0.0481	0.1419
$C_{16:0}$	7.3213	10.6772	3.9496	3.8797	4.1826	10.7381
$C_{16:1}$	0.3434	0.6230	0.1360	0.1408	0.2025	0.6661
$C_{18:0}$	**4.1630**	2.9979	2.1986	2.6323	3.0341	**7.5048**
$C_{18:1}$	42.6632	28.1662	72.7333	72.5224	70.0784	31.4668
$C_{18:2}$	29.2415	37.0096	**11.5253**	**11.4008**	**12.2550**	29.4236
$C_{18:3}$	9.5494	12.4735	4.5227	4.6344	5.1038	10.4718
$C_{20:0}$	**1.7108**	**1.5811**	1.1132	1.2070	1.3821	**2.6041**
$C_{20:1}$	2.2521	2.4504	1.7807	1.6792	1.6406	2.6668
$C_{20:2}$	0.1620	0.1600	0.0726	0.0724	0.0700	0.1295
$C_{22:0}$	1.1188	1.7861	0.9534	0.9449	0.9628	1.8995
$C_{22:1}$	0.1219	0.2030	0.1075	0.0875	0.0000	0.0000
$C_{24:0}$	0.7574	0.9286	0.5629	0.5129	0.6498	1.6442
$C_{24:1}$	0.5197	0.8334	0.3071	0.2465	0.3903	0.6428
FDA	**15.1468**	**18.0809**	8.8148	9.2161	10.2594	**24.5325**

[1]line designation; [2]%

Example 8—T3 plants and T4 seeds

About 0.5 g of T3 seed from each T2 plant selected for advancement as described in Example 7, were planted in field plots in Colorado, USA. T4 seeds were collected and combined from 20 random T3 plants from each line and analyzed for fatty acid composition (by GC; see Example 3) and oil content (by NMR; see Example 4). Following random bulk T4 seed analysis from each plot, 5-10 T4 seeds from those lines exhibiting increased oil content were advanced individually in the greenhouse.

Thirteen T3 lines with oil content of 48.7% to 50% were advanced and one T3 line with oil content of 48.1% was advanced from the -7ACCase/Westar transformation. Table 6 shows summary statistics (mean ± standard deviation) for seed fatty acid profiles of the total T4 population, the plants selected for advancement and corresponding non-transgenic control plants. Data for the non-transgenic control population was obtained from 139 Westar plants transgenic for an *fae1* gene. The *fae1* gene elongates $C_{18:1}$ to $C_{20:1}$, thereby resulting in an accumulation of $C_{20:1}$ in plants transgenic for *fae1*, but does not affect oil content.

TABLE 6

Mean fatty acid profile and oil content of T4 seeds

Fatty acid	-7ACCase/Westar		
	Total[1]	Sel[1]	Control[1]
$C_{14:0}$	0.06[2] (0.01)	0.06 (0.01)	0.07 (0.03)
$C_{16:0}$	3.52 (0.40)	3.52 (0.12)	3.48 (0.52)
$C_{16:1}$	0.19 (0.03)	0.18 (0.01)	0.19 (0.05)
$C_{18:0}$	3.00 (8.76)	2.12 (0.13)	2.15 (0.24)
$C_{18:1}$	68.58 (6.64)	69.83 (0.33)	64.60 (8.61)
$C_{18:2}$	15.26 (1.98)	15.30 (0.44)	14.68 (1.32)
$C_{18:3}$	6.42 (0.90)	6.40 (0.29)	6.49 (0.58)
$C_{20:0}$	0.71 (0.11)	0.66 (0.01)	0.75 (0.18)
$C_{20:1}$	1.26 (0.19)	1.18 (0.05)	5.47 (7.83)
$C_{20:2}$	0.05 (0.01)	0.05 (0.00)	0.14 (0.18)
$C_{22:0}$	0.34 (0.04)	0.29 (0.01)	0.32 (0.04)
$C_{22:1}$	0.08 (0.44)	0.05 (0.08)	0.92 (2.23)
$C_{24:0}$	0.30 (0.29)	0.22 (0.04)	0.22 (0.09)
$C_{24:1}$	0.24 (0.25)	0.15 (0.09)	0.52 (0.57)

FDA	7.92 (8.58)	6.86 (0.16)	6.98 (0.65)
% Oil	45.7 (3.2)	49.1 (0.51)	45.5 (1.92)

[1]Total, mean composition of all T3 plants; Sel, mean composition of T3 plants selected for advancement; Control, mean composition of non-transformed Westar plants; [2]mean in percent (± standard deviation).

Table 7 shows the fatty acid profiles of T4 seed from representative individual lines from the -7ACCase/Westar transformation that were selected for advancement.

TABLE 7

Fatty acid profile and oil content of T4 seeds from representative -7ACCase/Westar lines selected for advancement

Fatty acid	001-31-07[1]	001-30-02	001-31-06	001-30-05
$C_{14:0}$	0.06[2]	0.06	0.06	0.07
$C_{16:0}$	3.59	3.52	3.42	3.70
$C_{16:1}$	0.19	0.17	0.17	0.18
$C_{18:0}$	2.01	2.10	2.07	2.10
$C_{18:1}$	69.76	69.47	70.12	69.49
$C_{18:2}$	15.64	16.21	15.08	15.49
$C_{18:3}$	6.24	5.99	6.48	6.45
$C_{20:0}$	0.65	0.65	0.66	0.67
$C_{20:1}$	1.13	1.14	1.18	1.15
$C_{20:2}$	0.04	0.05	0.05	0.05
$C_{22:0}$	0.30	0.28	0.30	0.29
$C_{22:1}$	0.02	0.02	0.02	0.01
$C_{24:0}$	0.23	0.23	0.26	0.23
$C_{24:1}$	0.14	0.12	0.14	0.12
FDA	6.84	6.84	6.76	7.06
%Oil	50.0	50.0	49.4	49.4

[1]line designation; [2]%

Example 9—T4 plants and T5 seeds

T4 seeds from 10 random selfed plants representing each line selected for advancement in Example 8 were planted in a greenhouse using 5-10 seeds per row. T4 plants were selfed, and T5 seeds were collected from individual plants. A portion of the T5

seeds from each line were combined and analyzed for oil content and fatty acid analysis by NIR as described in Example 5.

Table 8 shows summary statistics (mean ± standard deviation) for seed oil and seed protein content for the total T5 population, for T5 lines selected for advancement and for corresponding non-transgenic controls. Data for the Westar control plants was obtained from 5 'control samples'. Each 'control sample' contained seed bulked from approximately 20 control plants.

Forty-five T4 plants (representing 6 lines from 14 plots) from the -7ACCase/Westar transformation yielded seed having an oil content of 44.4% to 50.4% and 7 of those plants, representing 2 lines (001-30-02 and 001-31-07) yielding seed having an oil content ranging from 44.4% to 50.1%, were advanced.

TABLE 8

Oil content, protein content and fatty acid profile of T5 seeds

Fatty acid	-7ACCase/Westar		
	Total[1]	Sel[1]	Control[1]
$C_{18:1}$	69.00[2] (0.90)	69.40 (0.60)	68.15 (0.56)
$C_{18:2}$	13.60 (1.30)	13.20 (29.00)	14.60 (0.68)
$C_{18:3}$	7.60 (0.60)	7.80 (0.30)	7.09 (0.50)
% Oil	49.90 (1.60)	49.90 (1.60)	45.64 (1.22)
% Protein	20.00 (1.10)	20.50 (1.20)	24.20 (0.22)
Chlor[3]	36.60 (18.20)	37.10 (25.60)	18.15 (4.86)
Gluc[4]	4.80 (1.00)	4.60 (1.10)	ND ND

[1]Total, mean composition of all T4 plants; Sel, mean composition of T4 plants selected for advancement; Control, mean composition of non-transformed Westar plants; [2]mean in percent (± standard deviation); [3]Chlorophyl content reported in parts per million (ppm); [4]Glucosinolate content reported in µmol/g; ND, not determined.

Table 9 shows the fatty acid profiles of T5 seed from representative individual lines from the -7ACCase/Westar transformation that were selected for advancement.

TABLE 9

Oil content, protein content and fatty acid profile of T5 seed from representative -7ACCase/Westar lines selected for advancement

	001-30-02[1]	001-30-02	001-30-02	001-31-07
$C_{18:1}$	69.0[2]	69.6	70.4	69.2
$C_{18:2}$	12.6	12.5	12.3	14.1
$C_{18:3}$	8.5	8.1	7.6	7.8
% Oil	50.3	50.2	49.8	52.3
% Protein	20.9	20.3	20.6	18.4
Chlor[3]	63.9	20.8	31.3	13.7
Gluc[4]	5.8	4.1	4.2	3.2

[1]line designation; [2]%

Example 10—T5 plants and T6 seeds

T5 lines that were selected based on percent oil and protein content as described in Example 9 were advanced in the field in Colorado, USA and in Saskatchewan, Canada. Approximately 0.5 g of seeds from each selected line were planted and selfed. At maturity, T6 seeds were collected from 20 plants of each line and pooled for analysis of oil content and fatty acid composition by NIR. Based upon NIR analysis and favorable oil content in the pooled sample of T6 seed, T6 seed from 10 random T5 plants from each line were advanced in the greenhouse.

Two lines from the -7ACCase/Westar transformation from T5 plants grown in Canada had T6 seeds that exhibited an oil content of 38.4% to 49.5%. The protein content in seeds harvested from the Canada-grown plants was measured in air-dried, oil-free seed meal (using the 'Generic Combustion Method for Determination of Crude Protein', AOCS Method Ba 4e-93), and the mean was determined to be 46.62% (±1.33) in T6 seed from the -7ACCase/Westar transformation. Percent protein content as shown in Table 10 for the Canadian samples was estimated based upon the percent protein content reported for the air-dried, oil-free seed meal using the oil content reported and assuming a moisture content of 5%.

T5 plants representing six lines of the -7ACCase/Westar transformation grown in Colorado, USA produced seed that had an oil content of 41.9% to 51.0%, and plants from five different lines, having an oil content of 48.8% to 50.5%, were advanced.

Table 10 shows the mean oil content (± standard deviation) of the T6 plants and control plants grown in Canada, and Table 11 shows the corresponding data for the total population of T6 plants grown in the USA, those T6 plants selected for advancement and from non-transgenic control plants grown in the USA. USA-grown controls for the -7ACCase/Westar transformation consisted of 2 control samples each of IMC129 and IMC130 (IMC129 and IMC130 are both related in the following way to the Westar variety: IMC129 carries a mutation and is otherwise ≥99% Westar background, while IMC130 is the result of a cross between IMC01 and IMC129 varieties, and therefore, contains ≤50% of the Westar background). Canadian-grown controls for the -7ACCase/Wester transformation consisted of 2 IMC130 control samples.

TABLE 10

Oil content, protein content and fatty acid profile of Canadian-grown T6 seeds

	-7ACCase/Westar	
	Total[1]	Control[1]
% Oil	46.78[2] (2.88)	42.69 (0.18)
% Protein	26.12[3]	26.66[3]

[1]Total, mean composition of all T5 plants; Control, mean composition of non-transformed Westar plants; [2]mean in percent (± standard deviation); [3]percent protein content was estimated based upon the percent protein content reported for the air-dried, oil-free seed meal using the oil content reported and assuming a moisture content of 5%.

TABLE 11

Oil content, protein content and fatty acid profile of USA-grown T6 seeds

	-7ACCase/Westar		
	Total[1]	Sel[1]	Control[1]
$C_{18:1}$	68.71[2] (1.64)	68.54 (0.77)	68.19 (6.76)
$C_{18:2}$	15.17 (1.26)	15.09 (0.78)	16.03 (5.12)
$C_{18:3}$	8.05 (0.56)	8.02 (0.23)	7.44 (2.36)

% Oil	47.84 (1.45)	49.48 (0.66)	48.54 (1.65)
% Protein	25.18 (1.45)	23.45 (1.09)	23.84 (1.90)
Chloro[3]	-3.59 (2.99)	-5.67 (1.02	-0.77 (3.35)
Gluc[4]	5.23 (0.64)	4.87 (0.69)	5.35 (1.24)

[1]Total, mean composition of all T6 plants; Sel, mean composition of T6 plants selected for advancement; Control, mean composition of non-transformed Westar plants; [2]mean in percent (± standard deviation); [3]Chlorophyll content reported in parts per million (ppm); [4]Glucosinolate content reported in μmol/g.

Using a one-tailed, two-sample Student's t-test, results from the T6 seeds were evaluated for statistical significance. The average oil content of the total T6 population from Canadian field plots was compared with the average oil content from the corresponding non-transgenic plants grown in Canada, while the T6 population selected for advancement from field plots in the USA was compared with the corresponding USA-grown control population for each line.

The -7ACCase/Westar plants grown in Canada (n=35) had an average oil content that was significantly higher ($p<0.1$) than that of the control population (n=2).

The -7ACCase/Westar plants grown in the USA and selected for advancement (n=17) had a higher oil content that was statistically significant ($p<0.05$) compared to the average oil content of the control plants (n=5).

Table 12 shows the percent oil content in T6 seeds from 6 representative individual lines selected for advancement from the -7ACCase/Westar transformation. Control plants grown in the field in Colorado, USA produced seeds that exhibited an average oil content of 44.62 (on a dry weight basis).

TABLE 12

Oil content of USA-grown T6 seeds from individual -7ACCase/Westar lines selected for advancement

Line	% Oil

Tao-001-30-02	48.86-49.58
Tao-001-31-02	49.93-50.51
Tao-001-56-01	48.86-49.42
Tao-001-56-06	48.84-50.39
Tao-001-65-08	49.10-49.85

Example 11—PCR analysis

A nickel size portion of leaf tissue was taken at 2.5 weeks post-germination from 12 T7 plants (representing 12 different –7ACCase/Westar transformed lines) grown from the T6 seeds described in Example 10. Tissue samples were dried in a food dehydrator at 135°C for 8-16 hrs. DNA was isolated using the Qiagen Dneasy 96 Plant Kit and resuspended in 150 µl buffer.

PCR amplification was performed in a volume of 20 µl containing the following: 1X PCR Buffer containing 1.5 mM $MgCl_2$ (Qiagen PCR Core Kit); 0.2 mM dNTP; 0.5 units Taq polymerase (Qiagen); 0.5 µM MF-ACCase 119 primer (5'-GTAGGCACCCTGCTACTACA (SEQ ID NO:9)); 0.5 µM MF-ACCase 645 primer (5'-CATCAGGAATAGTAATCAAGTCA (SEQ ID NO:10)); 0.4% sucrose; 0.008% Cresol. A 30 cycle amplification was performed using the following PCR conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 60 sec. PCR products were analyzed by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products of the predicted size were detected in all 12 -7ACCase/Westar plants analyzed, indicating the presence of the alfalfa cytosolic ACCase gene in all lines examined.

Example 12—Crosses between the T6 plants

T6 seeds from the selected lines shown in Table 12 were grown in the field in Colorado, USA. Reciprocal crosses were made between the T6 plants derived from the -7ACCase/Westar transformation (lacking a transit peptide) and two T6 plants derived from a +6ACCase/IMC 03 transformation (having a transit peptide). Plants were grown to maturity and the seeds were harvested. F1 seeds are grown and the resulting plants are allowed to self-pollinate. The resulting F2 progeny seeds are harvested, and the fatty acid profile is determined and oil and protein content are analyzed as described in Examples 3-5. Samples exhibiting a statistically significant increase in oil content are selected for advancement.

Example 13—Outcrosses between the T6 plants and other plant varieties

T6 seeds from the selected lines shown in Table 12 were grown in the field in Colorado, USA. Crosses were made between the T6 plants derived from the -7ACCase/Westar transformation (lacking a transit peptide) or two T6 plants derived from a +6ACCase/IMC 03 transformation (having a transit peptide) and plants of a *Brassica* line exhibiting high oleic acid content. An example of a high oleic acid *Brassica* variety is Q4275, described in PCT 96/20090. F1 seeds are grown and the resulting plants are allowed to self-pollinate. The resulting F2 progeny seeds are harvested, and the fatty acid profile is determined and oil and protein content are analyzed as described in Examples 3-5. Samples exhibiting a statistically significant increase in oil content, as well as high oleic acid content, are selected for advancement.

T6 seeds from the selected lines shown in Table 12 were grown in the field in Colorado, USA. Crosses were made between the T6 plants derived from the -7ACCase/ Westar transformation (lacking a transit peptide) or two T6 plants derived from a +6ACCase/IMC 03 transformation (having a transit peptide) and plants of a *Brassica* line exhibiting elevated oil content but lacking an ACCase construct. Examples of *Brassica* varieties exhibiting elevated oil are IMC106RR and IMC107RR, proprietary Cargill *Brassica* lines. The oil content in IMC106RR or IMC107RR is about 46.5-47% and 47.5-48%, respectively, on a dry weight basis. Another example of a *Brassica* line that exhibits elevated oil content is Polo, a non-transgenic variety registered in Canada (Registration # AG012). Polo has an oil content of about 48.5-49.5% on a dry weight basis. F1 seeds were grown in the greenhouse and the resulting plants allowed to self-pollinate. The resulting F2 progeny seeds are harvested, and the fatty acid profile is determined and oil and protein content are analyzed as described in Examples 3-5. Seeds exhibiting an oil content that is significantly higher than either parental line are selected for advancement. Progeny plants are allowed to self-pollinate and the seeds analyzed for oil content. Those seeds exhibiting increased oil content are advanced.

T6 seeds from the selected lines shown in Table 12 were grown in the field in Colorado, USA. Crosses were made between the T6 plants derived from the -7ACCase/ Westar transformation (lacking a transit peptide) or two T6 plants derived from a

+6ACCase/IMC 03 transformation (having a transit peptide) and plants of a *Brassica* line exhibiting high erucic acid content but lacking an ACCase construct. Suitable high erucic acid *Brassica* lines include, for example, Hero (HE101, HEC01), Mercury, Venus or Neptune which have about 45% or more erucic acid (McVetty et al., *Can. J. Plant Sci.*, 76(2):341-342 (1996); Scarth et al., *Can. J. Plant Sci.*, 75(1):205-206 (1995); and McVetty et al., *Can J. Plant Sci.*, 76(2):343-344 (1996)). F1 seeds were grown in the greenhouse and the resulting plants allowed to self-pollinate. The resulting F2 progeny seeds are harvested, and the fatty acid profile is determined and oil and protein content are analyzed as described in Examples 3-5. Seeds exhibiting an oil content that is significantly higher than either parental line are selected for advancement. Progeny plants are allowed to self-pollinate and the seeds analyzed for oil content. Those seeds exhibiting increased oil content are advanced.

Additionally, PCR is used to examine the segregation of the alfalfa ACCase nucleic acid in the progeny of the above-described crosses. After crossing T6 plants derived from a -7ACCase/Westar transformation or T6 plants derived from a +6ACCase/IMC 03 transformation with an appropriate plant (*i.e.*, a plant exhibiting high oil, high oleic acid or high erucic acid), F1 seeds are harvested, grown in the greenhouse and the resulting plants are allowed to self-pollinate. The resulting F2 progeny seeds are harvested, and PCR is performed to detect the presence of the alfalfa ACCase nucleic acid sequences using DNA extracted from the seed. Alternatively, the F2 seeds are grown into mature F3 plants, and PCR is performed, using DNA extracted from the leaves of the plant to detect the presence of the alfalfa ACCase nucleic acid sequences. Representative PCR primers homologous to the alfalfa ACCase are described in Example 11. If PCR amplification indicates the presence of the alfalfa ACCase nucleic acid sequences, oil content is then determined by NMR or NIR as described in Examples 4 and 5. Seeds or plants are subsequently advanced based upon a positive PCR amplification (*i.e.*, the presence of the alfalfa ACCase nucleic acid sequences) and elevated oil content.

Example 14 - Increased oil content in crushed seeds

T6 seeds of Example 10 are planted, allowed to pollinate, and the resulting seeds are harvested and crushed. The oil content of the crushed seeds is about 5% to about 25% higher than the oil content in a corresponding plant lacking an ACCase construct. The oil is

extracted from the crushed seeds as described in, *e.g.*, U.S. Patent No. 5,969,169 or 5,850,026.

Briefly, the seed is cleaned through commercial seed cleaning equipment to remove foreign matter such as weed seeds, plant material, immature seed and other matter. The cleaned seed is crushed and the resulting oil is processed at the Cargill Plant (West Fargo, ND). Greater than 350 tons of seed is crushed using the processing conditions outlined below.

Whole seed is passed through a double roll Bauermeister flaking rolls with smooth surface rolls available from Bauermeister Inc. (Memphis, TN). The roll gap is adjusted so as to produce a flake 0.25 to 0.30 mm in thickness. Flaked seed is conveyed to a five-tray, 8-foot diameter stacked cooker, manufactured by Crown Iron Works (Minneapolis, MN). The flaked seed moisture is adjusted in the stacked cooker to 5.5-6.0%. Indirect heat from the steam heated cooker trays is used to progressively increase the seed flake temperature to 80-90°C, with a retention time of approximately 20-30 minutes. A mechanical sweep arm in the stacked cooker is used to ensure uniform heating of the seed flakes. Heat is applied to the flakes to deactivate enzymes, facilitate further cell rupturing, coalesce the oil droplets and agglomerate protein particles in order to ease the extraction process.

Heated flakes are conveyed to a screw press from Anderson International Corp. (Cleveland, OH) equipped with a suitable screwworm assembly to reduce press out of the oil from the flakes by approximately 70%. The resulting press cake contains a small percentage of residual oil. Crude oil produced from the pressing operation is passed through a settling tank with a slotted wire drainage top to remove the solids expressed out with the oil in the screw pressing operation. The clarified oil is passed through a plate and frame filter to remove the remaining fine solid particles. The filtered oil is combined with the oil recovered from the extraction process before oil refining.

The press cake produced from the screw pressing operation is transferred to a FOMM basket extractor available from French Oil Mill and Machinery Co. (Piqua, OH) where the oil remaining in the cake is extracted with commercial *n*-hexane at 55°C. Multiple counter-current hexane washes are used to substantially remove the remaining oil in the press cake, resulting in a press cake that contains residual oil in the extracted cake. The oil and hexane mixture (miscella) from the extraction process is passed through a two-stage rising film tube

type distillation column to distill the hexane from the oil. Final hexane removal from the oil is achieved by passing the oil through a stripper column containing disk and doughnut internals under 23-26 in Hg vacuum and at 107-115°C. A small amount of stripping steam is used to facilitate the hexane removal. The oil recovered from the extraction process is combined with the filtered oil from the screw pressing operation, resulting in blended crude oil, and is transferred to oil processing.

In the oil processing, the crude oil is heated to 66°C in a batch-refining tank, to which 0.15% food-grade phosphoric acid, as 85% phosphoric acid, is added. The acid serves to convert the non-hydratable phosphatides to a hydratable form, and to chelate minor metals that are present in the crude oil. The phosphatides and the metal salts are removed from the oil along with the soapstock. After mixing for 60 minutes at 66°C, the oil acid mixture is treated with sufficient sodium hydroxide solution to neutralize the free fatty acids and the phosphoric acid in the acid oil mixture. This mixture is heated to 71°C and mixed for 35 minutes. The agitation is stopped and the neutralized free fatty acids, phosphatides, etc. (soapstock) are allowed to settle into the cone bottom of the refining tank for 6 hours. After the settling period, the soapstock is drained off from the neutralized oil.

A water wash is done to reduce the soap content of the oil by heating the oil to 82°C and adding 12% hot water. Agitation of the mixture continues for 10 minutes. The mixture is allowed to settle out for 4 hours, at which time the water is drained off the bottom of the refining vessel. The water washed oil is heated to 104-110°C in a vacuum bleacher vessel maintained at 24-26 in. Hg vacuum. A slurry of the oil and Clarion 470 bleaching clay available from American Colloid Company (Refining Chemicals Division, Arlington Heights, IL) is added to the oil in the vacuum bleacher. This mixture is agitated for 20 minutes before filtering to remove the bleaching clay. The clay slurry addition is adjusted to provide a Lovibond color (AOCS Official Method Cc 136-4) of less than 1.0 red units when the oil is heated to 288°C under atmospheric pressure. Nitrogen is injected into the filtered bleached oil and is maintained under a nitrogen blanket until the oil is deodorized.

Refined and bleached oil is deodorized in a semi-continuous Votator deodorizer tower at a rate of approximately 7,000 pounds per hour. The deodorization temperature is maintained at 265-268°C with a system pressure of 0.3-0.5 mm Hg absolute pressure. Sparge steam is used to strip off the free fatty acids, color bodies, and odor components.

Retention time in the deodorizer is generally 30-90 minutes. The deodorized oil is cooled to 45-50° C and nitrogen is injected prior to removal of the vacuum. The deodorized oil is stored under a nitrogen blanket and the resulting deodorized oil analyzed for fatty acid composition.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.